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Purification and characterization of the recombinant CMP-sialic acid synthetase from Neisseria meningitidis

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CMP-Sialic acid synthetase from Neisseria meningitidis 406Y was expressed in Escherichia coli K113 pLysS and produced at 360 U/L. The purified CMP-sialic acid synthetase used both N-acetyl-neuraminic acid (K_m = 0.34 mM) and N-glycolyl-neuraminic acid (K_m = 2.6 mM) as substrates. The recombinant synthetase could be used in a coupled reaction with an α-2,3-sialyltransferase to sialylate a lactose derivative in a one-reactor synthesis.

Introduction

The CMP-sialic acid (CMP-Neu5Ac) synthetase (EC 2.7.7.43) catalyses the following reaction:

$$CTP + Neu5Ac \rightarrow CMP\text{-}Neu5Ac + PP_i$$

CMP-Neu5Ac is a donor substrate for sialyltransferases which attach sialic acid to acceptor hydroxyl groups in various biopolymers including polysialic acids, glycolipids and glycoproteins (Tsuji, 1996). Since CMP-Neu5Ac is unstable and relatively expensive, the CMP-Neu5Ac synthetase is valuable for the preparative enzymatic synthesis of sialylated oligosaccharides. It can also be used to charge sialic acid analogs in order to synthesize the corresponding sialo-oligosaccharide analogs. Sialic acid activation has been reviewed by Kean (1991) and CMP-Neu5Ac synthetases have been isolated from various eukaryotic and prokaryotic sources. Several bacterial pathogens have been shown to possess sialylated capsular and lipo-polysaccharides as important virulence factors and this has motivated the study of sialic acid biosynthesis and incorporation in these organisms. *Neisseria meningitidis* was shown to be a good source of CMP-Neu5Ac synthetase by Warren and Blacklow (1962) but a non-pathogenic recombinant strain would be preferable for the scale-up of the production of this enzyme and its application in preparative syntheses of sialylated oligosaccharides. Bacterial genes encoding CMP-Neu5Ac synthetase have been cloned from *Escherichia coli* (Vann *et al.*, 1987), *Neisseria meningitidis* (Edwards and Frosch, 1992, Ganguli *et al.*, 1994), *Streptococcus agalactiae* (Haft *et al.*, 1996) and *Haemophilus ducreyi* (Tullius *et al.*, 1996). We report the production, purification and characterization of the recombinant *N. meningitidis* CMP-Neu5Ac synthetase and its use in a coupled reaction with an α-2,3-sialyltransferase.

Materials and methods

Cloning of the CMP-Neu5Ac synthetase gene

Based on the CMP-Neu5Ac synthetase sequence published by Ganguli *et al.* (1994), we designed the following oligonucleotides: 5′-CTTAGGAGGTCA-TATGGA-AAAACAAAATATTGCGGTTATAC-3′ (SYNTM-F1) and 5′-TCGAAGATCTCA-TCAGTG-GTGGTGGTGGTGGTTCAGGTCTTCTTCGCT-GATCAGTTTTTGTTCGCTTTCCTTGTGATTAAG AATGTTTTC-3′ (SYNTM-R2). SYNTM-F1 (5′ end primer) included a *Nde*I site while SYNTM-R2 (3′ end primer) included a *Bgl*II site and sequences encoding an epitope tag (c-myc) for immunodetection and a tail of 5 His residues for purification of the product by immobilized metal affinity chromatography. The PCR reaction was performed using a Perkin Elmer (Norwalk, CT, USA) GeneAmp PCR System 9600 and the 50 µL mix included 100 ng of chromosomal DNA from *N. meningitidis* 406Y, 20 pmoles of each primer, 0.5 mM dNTP, 2 mM $MgSO_4$ and 1 U of Vent DNA polymerase (New England Biolabs, Mississauga, Canada) in 1X buffer provided by the supplier. The PCR product was digested with *Nde*I and *Bgl*II and cloned in pT7–7 (Tabor and Richardson, 1985) cut with *Nde*I and *Bam*HI to obtain the plasmid pNSY-02.

Expression of the CMP-Neu5Ac synthetase

The plasmid pNSY-02 was transformed in *E. coli* K113 pLysS and an isolated colony was used to inoculate a 1 L culture containing 2X YT medium (16 g

EXHIBIT E

Bactotryptone, 10 g yeast extract, 5 g NaCl and H_2O to 1 L) with 150 μg/mL ampicillin and 30 μg/mL chloramphenicol. The 1 L culture was grown overnight at 37°C and used to inoculate 20 L of 2X YT with 150 μg/mL ampicillin. The 21 L culture was grown at 37°C in a 28-L New Brunswick Scientific (Edison, NJ, USA) fermenter (model MF 128S) until A_{600} = 0.6 and was then induced with 1 mM IPTG. The cells were collected 4 hours after the induction and the cell yield was 133 g (wet weight).

Purification of the CMP-Neu5Ac synthetase

Frozen cells, 43.3 g, were resuspended in 160 mL buffer (20 mM Tris; pH 8; 1 mM DTT; 50 μg DNAse I/mL) and left for 30 min on ice to allow lysis by the endogenous lysozyme. The extract was centrifuged for 30 min at 31,000 × g and streptomycin sulfate was then added to the supernatant to give 1.5% (w/v). The precipitated nucleic acids were removed by centrifugation (20 min, 20,000 × g) and the supernatant was applied to a 26 mm × 18 cm Q-Sepharose FF column (Pharmacia Biotech, Uppsala, Sweden). The Q-Sepharose column was developed with a 0–400 mM NaCl linear gradient (in 20 mM Tris, pH 8) and the most active fractions (230–330 mM NaCl) were applied (in 3 separate runs) to a 5 mL HiTrap Chelating column (Pharmacia Biotech) charged with Ni^{2+}. The column was developed with a 0–125 mM imidazole gradient in 10 mM HEPES (pH 7) containing 0.5 M NaCl.

N-terminal amino acid sequencing and molecular mass determination

Automated gas-phase amino acid sequencing was performed on an Applied Bio-systems (Foster City, CA, USA) 475A protein sequencing system incorporating a model 470A gas-phase sequencer equipped with an on-line model 120A PTH analyzer under the control of a model 900A control and data analysis module. Mass analysis was performed using a Fisons Instruments (Manchester, U.K.) VG electrospray Quattro triple quadrupole mass spectrometer with a mass range of 3500 amu/e. Solutions to be analyzed were prepared in 5% (w/v) acetic acid at 0.1–0.2 mg/mL and infused directly into the mass spectrometer. SDS-PAGE was performed according to Laemmli (1970).

Measurement of CMP-Neu5Ac synthetase activity

The reaction mix for the CMP-Neu5Ac synthetase assay included 0.2 M Tris (pH 8.5), 0.2 mM DTT, 20 mM $MgCl_2$, 3 mM Neu5Ac and 5 mM CTP. For K_m determination the concentration of Neu5Ac, Neu5Gc or CTP varied from 0.2 mM to 10 mM. The assay was performed in a total volume of 50 μL for 10 min. at 37°C and stopped by the addition of 5 μL of 0.25 M EDTA. The reaction mix was analyzed by capillary electrophoresis using a Beckman Instruments (Fullerton, CA) P/ACE 5000 and monitoring the eluate at 214 nm. The capillary was bare silica 75 μm × 47 cm with the detector at 40 cm and the running buffer was 25 mM sodium tetraborate, pH 9.4. One unit of activity was defined as the amount of enzyme that produces one (mol of CMP-Neu5Ac in one minute.

Sialylation of FCHASE-lactose

The acceptor oligosaccharide in the coupled sialylation reaction was aminophenyl-lactose-6-(5-fluorescein-carboxamido)-hexanoic acid amide (FCHASE-lactose). The reaction mix included 0.5 mM FCHASE-lactose, 2.5 mM Neu5Ac, 2.5 mM CTP, 0.1 M Tris (pH 7.5), 0.2 mM DTT, 10 mM $MnCl_2$, 10 mM $MgCl_2$, 0.2% Triton X-100, 13 mU of *N. meningitidis* α-2,3-sialyltransferase (Gilbert *et al.*, 1996) and 47 mU of CMP-Neu5Ac synthetase. The reaction was performed at 32°C for 60 min and the production of sialylated FCHASE-lactose was followed by capillary electrophoresis using a Beckman Instruments P/ACE 5510 equipped with a 3 mW Argon-ion laser-induced fluorescent detector. The capillary electrophoresis running conditions were as described by Gilbert *et al.* (1996).

Results and discussion

Cloning of the CMP-Neu5Ac synthetase gene from N. meningitidis 406Y

Using chromosal DNA from *N. meningitidis* 406Y and the primers SYNTM-F1 and SYNTM-R2, we obtained a PCR product of the expected size (0.82 kb) and cloned it in pT7–7 giving the construct pNSY-02. We sequenced the insert of 2 distinct clones and confirmed that the sequence of the PCR product corresponds to the CMP-Neu5Ac synthetase gene (GeneBank accession No. U60146). We found a single base difference (nt 585 is T instead of C) from the gene cloned by Ganguli *et al.* (1994, GeneBank accession No. X78068). This base substitution has no impact on the protein sequence and could be a PCR artifact but could also be a consequence of divergence between the *N. meningitidis* 406Y strain which has a Y-type capsule and the *N. meningitidis* strain used by Ganguli *et al.* (1994) which has a B-type capsule. The sequencing also showed a missing base in the 3′ sequence encoding the His-tail. The recombinant CMP-Neu5Ac synthetase has a 4-His tail and 5 additional residues at C-terminus instead of having the expected 5-His tail. However, these additional residues at the C-terminus did not appear to have

an effect on the CMP-Neu5Ac synthetase since it was active and could be purified using immobilized metal affinity chromatography (see below).

Production and purification of the CMP-Neu5Ac synthetase

The plasmid pNSY-02 was transformed into *E. coli* K113 pLysS which has a copy of the T7 RNA polymerase gene under the control of P_{lac} as well as a copy of the gene encoding T7 lysozyme. For the production of CMP-Neu5Ac synthetase, *E. coli* K113 pLysS (pNSY-02) was grown in a 21 L culture and induced with 1 mM IPTG. Analysis of cell extracts indicated a production of 360 units of CMP-Neu5Ac synthetase per liter of culture. The CMP-Neu5Ac synthetase was purified by a sequence of anion-exchange chromatography and immobilized metal affinity chromatography (IMAC) on a column charged with Ni^{2+}. The purified CMP-Neu5Ac synthetase was eluted from the IMAC column with 75–85 mM imidazole. SDS-PAGE analysis showed that the purified CMP-Neu5Ac synthetase was at least 95% homogenous (data not shown). From 7 L of culture we purified 587.2 units of CMP-Neu5Ac synthetase (specific activity of 69 U/mg) with an overall yield of 23.6%. Warren and Blacklow (1962) reported a specific activity of 121 U/mg for the native CMP-Neu5Ac synthetase purified from *N. meningitidis* cells but a direct comparison with the recombinant CMP-Neu5Ac synthetase is not possible since they used different methods for assaying the protein concentration and the enzymatic activity.

Characterization of the purified CMP-Neu5Ac synthetase

N-Terminal sequencing indicated that the first residue of the recombinant CMP-Neu5Ac synthetase was unformylated Met. We also sequenced the next 40 residues which were in perfect agreement with the ones expected based on the DNA sequence. Using electrospray mass spectrometry we measured a molecular mass of 27,313.24 Da which is close to the molecular mass predicted from the translated DNA sequence (27,313.12 Da). However, the CMP-Neu5Ac synthetase displayed anomalously slow migration on SDS-PAGE, having an apparent molecular mass of 33,700 Da on a 12% (w/v) acrylamide gel.

The K_m values for CTP and Neu5Ac were 0.31 mM and 0.34 mM, respectively. We observed that the recombinant CMP-Neu5Ac synthetase could also use *N*-glycolyl-neuramininic acid (Neu5Gc) as a substrate although the K_m (2.6 mM) was 7.6-fold higher than with Neu5Ac. Despite this K_m difference, both substrates showed comparable k_{cat} values of 36 sec^{-1} (Neu5Gc) and 32 sec^{-1} (Neu5Ac). Warren and Blacklow (1962) reported that the CMP-Neu5Ac synthetase purified from *N. meningitidis* could not use Neu5Gc as a substrate. The reason for the discrepancy between our observation that the CMP-Neu5Ac synthetase can use Neu5Gc and the result reported by Warren and Blacklow (1962) is unclear but they might have failed to observe low levels of product if the Neu5Gc concentration used in their assay was well below the K_m of 2.6 mM.

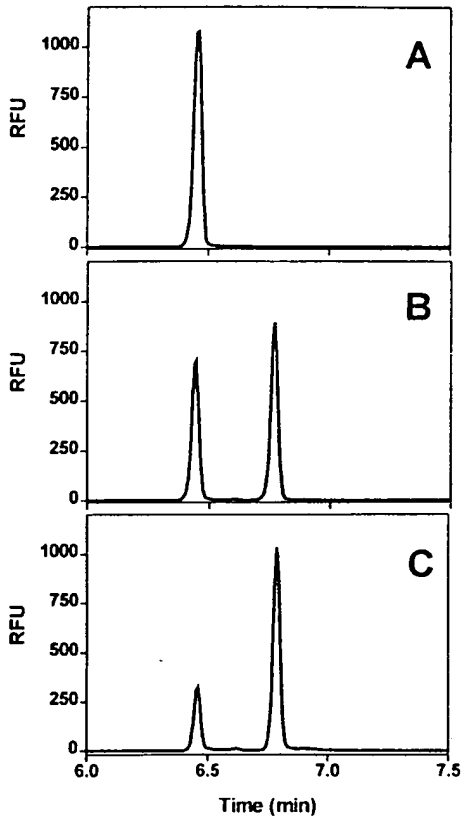

Figure 1 Sialylation of FCHASE-lactose using a coupled reaction with the N. meningitidis CMP-Neu5Ac synthetase and α-2,3-sialyltransferase. The reaction was followed by capillary electrophoresis using laser-induced fluorescence detection. The detector response is in relative fluorescence units (RFU). The acceptor eluted at 6.45 min and the sialylated product eluted at 6.78 min. Panel A: before the addition of the CMP-Neu5Ac synthetase. Panel B: 10 min after the addition of the CMP-Neu5Ac synthetase. Panel C: 60 min after the addition of the CMP-Neu5Ac synthetase.

Sialylation of FCHASE-lactose using a coupled reaction

A major application of the CMP-Neu5Ac synthetase is in coupled reactions with sialyltransferases to sialylate oligosaccharides using CTP and Neu5Ac as substrates instead of CMP-Neu5Ac which is relatively unstable and expensive. We used the CMP-Neu5Ac synthetase and the α-2,3-sialyltransferase from *N. meningitidis* (Gilbert *et al.*, 1996) in a coupled reaction to sialylate FCHASE-lactose (Fig. 1). After 60 minutes, 75% of the FCHASE-lactose was converted to its sialylated form, which indicated that the recombinant CMP-Neu5Ac synthetase from *N. meningitidis* can work effectively in a coupled reaction with a sialyltransferase.

The recombinant CMP-Neu5Ac synthetase reported in this work displays the properties required for its use in preparative syntheses of sialylated oligosaccharides. The production level under simple culture conditions is high (360 U/L) and it can be purified easily. It was shown to use Neu5Gc as a substrate which indicates that it does not have a strict specificity for Neu5Ac and could possibly accept other sialic acid analogs. It was also shown to work in a coupled reaction with a sialyltransferase to sialylate an oligosaccharide using CTP and Neu5Ac as substrates.

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References

Edwards, U. and Frosch, M. (1992). *FEMS Microbiol. Lett.* 96, 161–166.

Ganguli, S., Zapata, G., Wallis, T., Reid, C., Boulnois, G., Vann, W.F. and Roberts, I.A. (1994). *J. Bacteriol.* 176, 4583–4589.

Gilbert, M., Watson, D.C., Cunningham, A.-M., Jennings, M.P., Young, N.M. and Wakarchuk, W.W. (1996). *J. Biol. Chem.* 271, 28271–28276.

Haft, R.F., Wessels, M.R., Mebane, M.F., Conaty, N. and Rubens, C.E. (1996). *Mol. Microbiol.* 19, 555–563.

Kean, E.L. (1991). *Glycobiol.* 1, 441–447.

Laemmli, U.K. (1970). *Nature* 227, 680–685.

Tabor, S. and Richardson, C.C. (1985). *Proc. Natl. Acad. Sci. USA* 82, 1074–1078.

Tsuji, S. (1996). *J. Biochem.* 120, 1–13.

Tullius, M.V., Munson Jr., R.S., Wang, J. and Gibson, B.W. (1996). *J. Biol. Chem.* 271, 15373–15380.

Vann, W.F., Silver, R.P., Abeijon, C., Chang, K., Aaronson, W., Sutton, A., Finn, C.W., Lindner, W. and Kotsatos, M. (1987). *J. Biol. Chem.* 262, 17556–17562.

Warren, L. and Blacklow, R.S. (1962). *J. Biol. Chem.* 237, 3527–3534.

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